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In vitro metabolism of T-2 mycotoxin (T-2) was studied in Vero cells, rat spleen lymphocytes, chicken embryo heart cells, rat small intestinal segments, and rat liver hepatocytes. The method used was thin-layer chromatography (TLC) of [^{3}H]T-2 and its metabolic products, followed by radioactive scanning of the plates. Vero cells, lymphocytes, and heart cells metabolized 5 to 35 percent of the T-2 to HT-2 mycotoxin (HT-2) after 24 hr exposure. No other metabolites were detected with these three cell systems. Rat intestinal segments, everted onto pipets, converted T-2 into three metabolites migrating in the range

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between T-2 tetraol and HT-2 on the TLC plates. Hepatocytes metabolized T-2 most rapidly, as indicated by complete disappearance of the parent compound within 4 hr. In addition to the T-2 peak, four predominant peaks appeared on the plates, one of them, increasing with time at the origin, was predominantly composed of glucuronide conjugates. Based on comparison of R_f values to standards, the other three were identified as 3'-hydroxy T-2, HT-2, and 3'-hydroxy HT-2. Media from hepatocytes or subcellular fractions of hepatocyte cultures metabolized T-2 slower than cells and the only metabolite detected was HT-2. These studies indicate that liver and intestine actively metabolize T-2 mycotoxin while lymphocytes and heart cells have little effect.

In Vitro Metabolism of T-2 Mycotoxin[1,2]

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In Vitro Metabolism of T-2 Mycotoxin. THOMPSON, W.L., PACE, J.G., AND O'BRIEN, J.C. (19). Fundam. Appl. Toxicol. , - . In vitro metabolism of T-2 mycotoxin (T-2) was studied in Vero cells, rat spleen lymphocytes, chicken embryo heart cells, rat small intestinal segments, and rat liver hepatocytes. The method used was thin-layer chromatography (TLC) of [^{3}H]T-2 and its metabolic products, followed by radioactive scanning of the plates. Vero cells, lymphocytes and heart cells metabolized 5 to 35 percent of the T-2 to HT-2 mycotoxin (HT-2) after 24 hr exposure. No other metabolites were detected with these three cell systems. Rat intestinal segments, everted onto pipets, converted T-2 into three metabolites migrating in the range between T-2 tetraol and HT-2 on the TLC plates. Hepatocytes metabolized T-2 most rapidly, as indicated by complete disappearance of the parent compound within 4 hr. In addition to the T-2 peak, four predominant peaks appeared on the plates, one of them, increasing with time at the origin, was predominantly composed of glucuronide conjugates. Based on comparison of R_f values to standards, the other three were identified as 3'-hydroxy T-2, HT-2, and 3'-hydroxy HT-2. Media from hepatocytes or subcellular fractions of hepatocyte cultures metabolized T-2 slower than cells and the only metabolite detected was HT-2. These studies indicate that liver and intestine actively metabolize T-2 mycotoxin while lymphocytes and heart cells have little effect.

T-2 mycotoxin, 4β,15-diacetoxy-8α-(3-methylbutyryloxy)-3α-hydroxy 12,13-epoxy-trichothec-9-ene (T-2), produced by Fusarium spp., is one of the most toxic of the trichothecene mycotoxins (Bamburg and Strong, 1971; Ueno et al., 1973). It is a potent inhibitor of protein synthesis in eukaryotic cells (Cannon et al., 1982; Oldham et al., 1980) with a 50% protein synthesis inhibition level in the ng range (Thompson and Wannnemacher, 1986). LD_{50}'s in several animal models have been established (Chan and Gentry, 1984; Thompson and Wannemacher, 1986) which are in the range of mg/kg body weight. Discrepancies in cellular cytotoxicity and whole animal lethality was due, at least in part, to the distribution, metabolism and clearance of the parent toxin. Several laboratories have described the distribution and metabolism of T-2 in animals such as mice and rats (Sato and Ueno, 1977; Matsumoto et al., 1978), broiler chickens (Yoshizawa et al., 1980a), cow (Yoshizawa et al., 1982) guinea pigs (Pace et al., 1985), and swine (Robison et al., 1979; Corley et al., 1985). Others have used embryonated hen's eggs (Bata et al., 1983), the S9 fraction from liver homogenates (Wei and Chu, 1985; Yoshizawa et al., 1980b and 1984; Ellison and Kotsonis, 1974), and cultured cells (Trusal, 1986). Some of the metabolites of T-2 described in these studies were shown by others to have reduced lymphocyte immunotoxicity (Forsell et al., 1985), whole animal lethality (Thompson and Wannemacher, 1986), and protein synthesis inhibition capability in cell cultures (Cundliffe and Davies, 1977; Thompson and Wannemacher, 1986; Ueno et al., 1973; Umeda, 1977). Recently, Watson and Hayes (1982) and Kravchenko, et al., (1984) have shown that the use of drugs that stimulate or suppress metabolic enzymes have an effect on the LD_{50} levels of some mycotoxins.

We looked at metabolism of [^{3}H]T-2 in an effort to better understand sites of T-2 metabolism in whole animal based on *in vitro* metabolic capabilities of cells from several tissues. The changing patterns of metabolites with time of exposure to these cells may provide clues to possible metabolic pathways of T-2 in various tissues of the animal.

MATERIALS AND METHODS

Cell Cultures. Seed stock for Vero tissue culture cells was obtained from the American Type Culture Collection (ATCC, Rockville, Md.). Before use, cells were grown to confluence as previously described (Thompson and Wannemacher, 1984).

Rat spleen lymphocytes were isolated and stimulated with concanavalin A (Sigma Chemical Co., St. Louis, Mo.) for 2 days prior to use, as previously described (Thompson and Wannemacher, 1984).

Rat intestinal segments were prepared by the procedures of Bronstein *et al.*, (1983) and Harrison and Webster, (1969), except that intestinal segments and not the released cells were used for metabolism studies. Briefly, 10 cm segments of rat small intestine (FD rats, 150-200 g) were cleaned and thoroughly rinsed in several changes of phosphate-buffered saline (PBS). Segments were tied to the end of 1 ml plastic pipettes with 3/0 silk (Deknatel, Queens Village, N.Y.), everted over the pipette, and the other end tied to a short segment of gum rubber tubing placed at the appropriate position on the pipette. The mounted segments were then gently rinsed several times with PBS and placed in a 10 ml sterile glass pipette with a piece of tubing and clamp

attached to the tip. Dulbecco's medium containing Ca^{2+} and Mg^{2+}, 10% fetal calf serum (KC Biologicals, Kansas City, Kans.) and 50 µg/ml gentamycin sulfate (Garamycin, Schering Corp., Kenilworth, N.J.) was added to each pipette and the intestinal segments placed in a CO_2 incubator several hours for equilibration.

Myocardial cells were isolated from 5 to 7 day-old chick embryos by the procedure of Ertell, et al. (1971) and set up in 6-well plates. Improvement in the proportion of myoblasts over endothelial cells was accomplished by including a differential attachment technique (Kasten, 1973) after isolation of the cells. Endothelial cells attach more rapidly to a glass surface, leaving a higher percentage of myoblasts in the media during a 90 min incubation: these can then be removed and plated. Cultures from this procedure reached confluence after 2 to 3 days, usually with 70% or more cells beating in synchrony.

Hepatocytes were isolated, by the collagenase procedure of Seglen (1972, 1973), with an MX Perfuser apparatus (MX International, Aurora, Colorado). After the perfusion with collagenase (type 1, Sigma Chemical Co., St. Louis, Mo.), cells were released into PBS by using a sterile metal dog-grooming comb, filtered through gauze and centrifuged at 50 x g for 2 min. After washing with PBS, cells were resuspended to a concentration of 5×10^5 cells/ml in William's E media containing 200 mM L-glutamate, 10 IU/ml insulin, 0.08 g/50 ml dexamethasone, 5.0 mM Hepes, and 5000 UI/ml Pen-Strep. Two ml aliquots of suspended cells were delivered to each well of 6-well plates. After allowing the cells to attach several hours, the medium was removed, replaced with fresh medium, and cells allowed to equilibrate overnight. In addition, medium was collected and centrifuged at various times after exposure to equilibrated

hepatocytes for tests of metabolism of T-2 by soluble enzymes released from hepatocytes. Hepatocytes were also scraped, homogenized with a Dounce homogenizer (Bellco Glass Inc., Vineland, N.J.) with a "b" pestle, and aliquots were centrifuged at 10K x g and 100K x g to study metabolism by subcellular fractions. Prior to T-2 addition, all fractions were diluted in Williams E suspension media at a concentration equivalent to their original cellular concentration.

T-2 Metabolism. [^{3}H]T-2, in the C3 position (Amersham Corp. Amersham, UK, sp act = 11 Ci/mmol), was added to each of the confluent monolayers or stabilized cell preparations at a concentration of 0.2 µg/ml by replacing the media in which the cells were cultured with media containing the mycotoxin. To study metabolism of T-2 by media exposed to hepatocytes and subcellular fractions of hepatocytes, T-2 was added to achieve the same final concentration (0.2 µg/ml). Aliquots of media containing labeled mycotoxin were collected at various times after CO_2 incubation at 37°C. After gentle inversion, approximately 0.5 ml of the intestinal segment medium was collected at each time period by release of the tubing clamp at the tip of the glass pipet. All samples were chilled rapidly in an ice bath, centrifuged at 650 x g for 5 min and the supernatants stored at 4°C. Ten µl aliquots of the supernatant were spotted on TLC plates. All but the Vero cell samples were chromatographed by using two sequential solvent systems containing chloroform:methanol:ethyl acetate, in the proportion 50:25:25 and 80:10:10, respectively. A mixture containing [^{3}H]T-2, HT-2, triol, and tetraol was also spotted to provide standard migration patterns for each experiment. The [^{3}H]-labeled standards were prepared by alkaline hydrolysis of labeled T-2 mycotoxin (Wei et al., 1971). R_f values for the 3'-hydroxy derivatives shown

in Table 1 were determined from standards supplied by Dr. F.S. Chu from the University of Wisconsin, Madison, Wis. The purity of all standards was greater than 95%, as determined by HPLC and GC/MS. After drying, the plates were run on a Bioscan BID200 radioisotopic scanner (Bioscan, Inc., Washington, D.C.) to determine position and quantity of T-2 and its metabolites.

RESULTS

Figures 1 through 6 show radiochromatograms produced by radioactive scanning of samples run on TLC plates. Samples were collected after various times of exposure of [^{3}H]T-2 to Vero tissue culture cells; four different primary cell types (lymphocytes, heart cells, intestinal cells, and hepatocytes); media exposed to hepatocytes; and subcellular fractions of hepatocytes. The concentration of T-2 chosen was based on recovery of sufficient counts in the media to produce good radioactive scans. It represents about a 20-fold excess over the normal in vitro 50% protein synthesis inhibition level of T-2 in most of these cell systems (Thompson and Wannemacher, 1986). In order to compare the various peaks which resulted from T-2 metabolism in the different cell systems, a summary table of R_f values is provided (Table 1).

No detectable metabolites of [^{3}H]T-2 were observed after 24 hr incubation at 37°C in medium only.

The rate of metabolism of T-2 in three of the cell types was very low. After 24 hr incubation with Vero cells, lymphocytes, and myoblasts, the amount of T-2 remaining was between 65 to 85 % (Figure 1). Generally, a reduced level of metabolism was observed after several trials with heart cells as compared to the other two cell types. The degree of T-2 metabolism in lymphocytes and Vero cells

was not statistically different at any time up to 24 hr. The single peak resulting from metabolism of T-2 exposed to these cells corresponds to the HT-2 standard. Unstimulated lymphocytes were also checked for metabolism of T-2 (not shown). No significant difference in the rate or amount of T-2 metabolism by stimulated or non-stimulated lymphocytes was noted.

Rat intestinal segments metabolized T-2 at a rate intermediate to lymphocytes and hepatocytes (Figure 2). Less than 9% of the T-2 remained by 24 hr. Four peaks were apparent at later time periods: T-2, HT-2 (R_f = 0.6, Table 1), and the last two in the area of the T-2 tetrasol standard (TMI-1 and TMI-2, R_f's = 0.31 and 0.37).

Metabolism of T-2 by intact hepatocytes (Figure 3) was also rapid but resulted in a different pattern of metabolites when compared to intestinal segments. T-2 was completely metabolized by 2 hr into four predominant peaks. The metabolite appearing initially in greatest quantity corresponded to HT-2. Two smaller peaks to either side of it co-migrating with 3'-hydroxy T-2 and 3'-hydroxy HT-2 standards (R_f's = 0.72 and 0.5), increased in quantity with time of exposure. Starting at 4 hr, the peaks corresponding to HT-2 and 3'-hydroxy T-2 began to diminish while the peak corresponding to 3'-hydroxy HT-2 increased along with a peak at the origin. The radioactive component remaining at the origin of the TLC plate was treated with β-glucuronidase (Limpets, type L-II, 1500 units/mg, pH 3.8, Sigma Chemical Co., St. Louis, Mo) by the procedure described by Pace et al. (1986), and re-processed on TLC plates (Figure 4). The peak remaining at the origin at the 24 hr time point contained 46% of the total radiolabel. Upon hydrolysis with β-glucuronidase, 6.1% of this peak remained at the origin. HT-2 (45%), 3'-hydroxy HT-2 (9.5%), and an unknown metabolite (20%, R_f = 0.42) were conjugated as glucuronides. Treatment of the material remaining at the origin with buffer only (control) resulted in retention of the original peak when rechromatographed on a TLC plate.

Therefore it appears that two end products of T-2 metabolism exist in hepatocytes. Glucuronide conjugates remaining at the origin, and one co-migrating with 3'-hydroxy HT-2 (Figure 3, Table 1).

Metabolism of T-2 by media which had previously been exposed to hepatocytes, and by subcellular fractions of hepatocytes is shown in Figures 5 and 6. The only metabolite in these systems was HT-2. The maximum amount of metabolism of T-2 in medium samples took place in media exposed to hepatocytes for 1 hr. The subcellular fraction having the most activity was the 10K X G pellet.

DISCUSSION

Results from these studies indicate that metabolism of T-2 differs significantly in various cell types. The level of activity demonstrated by Vero cells, lymphocytes, and heart cells is probably not sufficient to have any effect on detoxication of T-2. This is particularly true since the only metabolic product detected with these cells, HT-2, has a cellular cytotoxicity (Ueno et al., 1973; Thompson and Wannemacher, 1986) and whole animal lethality (Thompson and Wannemacher, 1986) only slightly less than T-2. Loss of metabolic activity in Vero cells was expected since there is a loss of differentiation of cells upon passage, resulting in lowered capability for metabolism. However, with freshly prepared cultures of myocardial cells and lymphocytes, any inherent capability for metabolism should still be relatively intact. The fact that cultured heart cells had the lowest rate of T-2 metabolism, even though they formed a confluent monolayer of cells beating in synchrony, may be of significance since the heart has been implicated as a possible site of T-2 lethality (Feuerstein et al., 1984; Pang et al., 1986; Yarom et al., 1983). The in vitro studies suggest that the heart might be more susceptible to the toxic effects of T-2 which had not been

previously metabolized and/or cleared by other tissues. Pronounced effects of the mycotoxins on lymphocytes and immunosuppression have been reported (Rosenstein et al., 1981; Yarom et al., 1984; Mann et al., 1982). No change in metabolic capability after lymphocyte proliferation suggests that stimulated lymphocytes had no increased protection against the effects of T-2, even though their protein and DNA synthesis levels were greatly enhanced by the mitogen.

From these studies, it appears that two of the most active tissues involved in metabolism or T-2 are intestine and liver. Due to their different metabolic products, it appears that they follow separate pathways of T-2 metabolism. Different metabolic pathways for T-2 metabolism by liver 9000 x g supernatant and rat intestinal strips (Yoshizawa, et al., 1980b) and formation of 3'-hydroxy derivatives of T-2 and HT-2 in the presence of NADPH (Yoshizawa, et al., 1984) and esterase inhibitors (Wei and Chu, 1985) have been proposed. These pathways correlate well with the time of appearance of various metabolites after T-2 exposure to hepatocytes. As depicted on Figure 7, T-2 is rapidly converted to HT-2, which then forms a glucuronide conjugate or, to a lesser degree, is further metabolized to 3'-hydroxy HT-2. A second pathway is a less-rapid metabolism of T-2 to 3'-hydroxy T-2, which then converts to 3'-hydroxy HT-2. The end products of these two pathways are the polar-glucuronide conjugates, which show up at the origin, and 3'-hydroxy HT-2. All of these pathways involve microsomal enzymes. Additional proposed minor pathways involving cytosolic enzymes and formation of 4- or 15-deacetyl-neosolaniol and T-2 tetraol were not involved in the metabolism of T-2 by hepatocytes. This was probably due to loss of cytosolic enzymes or their substrates during isolation and stabilization of hepatocytes. The unidentified products of T-2 metabolism by intestinal strips may also be the result of a minor pathway or one that has not yet been described.

The necessity of an intact cell system for complete metabolism of T-2 was demonstrated by our studies of hepatocyte media and cell fractions. The appearance of HT-2 as the only metabolite of T-2 in a cell-free system is in agreement with several investigators who used liver-cell subfractions (Ellison and Kotsonis, 1974; Ohtu, et al., 1977) but not with others (Yoshizawa, et al., 1980b, 1984). The latter studies yielded metabolites formed by cytosolic enzymes which were lacking even in our intact hepatocytes.

These studies demonstrate the value of using cell culture systems for predicting degrees of metabolism, metabolic products, and pathways utilized by a particular tissue. They also demonstrate the limitations of in vitro cell metabolism studies. Results must be carefully interpreted and supplemented with in vivo studies, since only a part of the metabolic pathways taking place in the animal may be represented in the cell systems. Future studies could involve the use of HPLC as a more sensitive method for detection of metabolites and mass spectrometry for specific identification of each metabolite.

Footnotes

[1] In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

[2] The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

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TABLE 1

TABLE OF R_f VALUES - T-2 metabolism in various cell types:

	Origin	TMI-2	T-2 Tetraol	TMI-1	T-2 Triol	3'OH HT-2	HT-2	Neosolaniol	3'OH T-2	T-2
STANDARDS	0.0		0.33		0.46	0.48	0.59	0.65	0.71	0.81
Vero cells							0.59			0.81
Lymphocytes							0.59			0.81
Heart cells							0.60			0.81
Intestinal segments		0.31		0.37			0.60			0.81
Hepatocytes	0.0					0.50	0.60		0.72	0.81

FIGURE LEGENDS

FIG. 1 Radiochromatograms of [^{3}H]T-2 mycotoxin after 24 hr exposure to Vero cells (A), 2-day concanavalin A-stimulated rat spleen lymphocytes (B) and rat heart myocardial cells (C). Numbers above the peaks represent the percent of total counts detected in each peak. The relative position of the peaks can be compared to mycotoxin standards (bottom scan) run at the same time. Relative counts = CPM detected by the Bioscan which represent 0.03% of the actual DPM.

FIG. 2 Radiochromatograms of [^{3}H]T-2 mycotoxin after 1, 4, 8, and 24 hr exposure to intestinal segments. Numbers above the peaks represent the percent of total counts detected in each peak. The relative position of the peaks can be compared to mycotoxin standards (bottom scan) run at the same time.

FIG. 3 Radiochromatograms of [^{3}H]T-2 mycotoxin after various times of exposure to rat hepatocytes. Numbers above the peaks represent the percent of total counts detected in each peak. The relative position of the peaks can be compared to mycotoxin standards (bottom scan) run at the same time.

FIG. 4 Treatment of T-2 metabolite appearing at the origin of a TLC plate with β-glucuronidase. A. TLC/Bioscan of 24 hr exposure of labeled T-2 to hepatocytes. B. TLC/Bioscan of origin fraction from "A" treated with buffer only. C. TLC/Bioscan of origin fraction from "A" treated with β-glucuronidase.

FIG. 5 Radiochromatogram of [^{3}H]T-2 mycotoxin after 3 hr exposure to media that had been previously exposed to hepatocytes for varying lengths of time (media pre-exposure time shown for each scan). The resulting two peaks on each scan corresponded to the position of T-2 (right peak) and HT-2 (left peak) when standards were run (not shown). Numbers above the peaks represent the percent of total counts detected in each peak.

FIG. 6 Radiochromatogram of [^{3}H]T-2 mycotoxin after a 4 hr exposure to various fractions from rat hepatocytes. The resulting two peaks correspond to the position of T-2 (right peak) and HT-2 (left peak) when standards were run (not shown). Numbers above the peaks represent the percent of total counts detected in each peak.

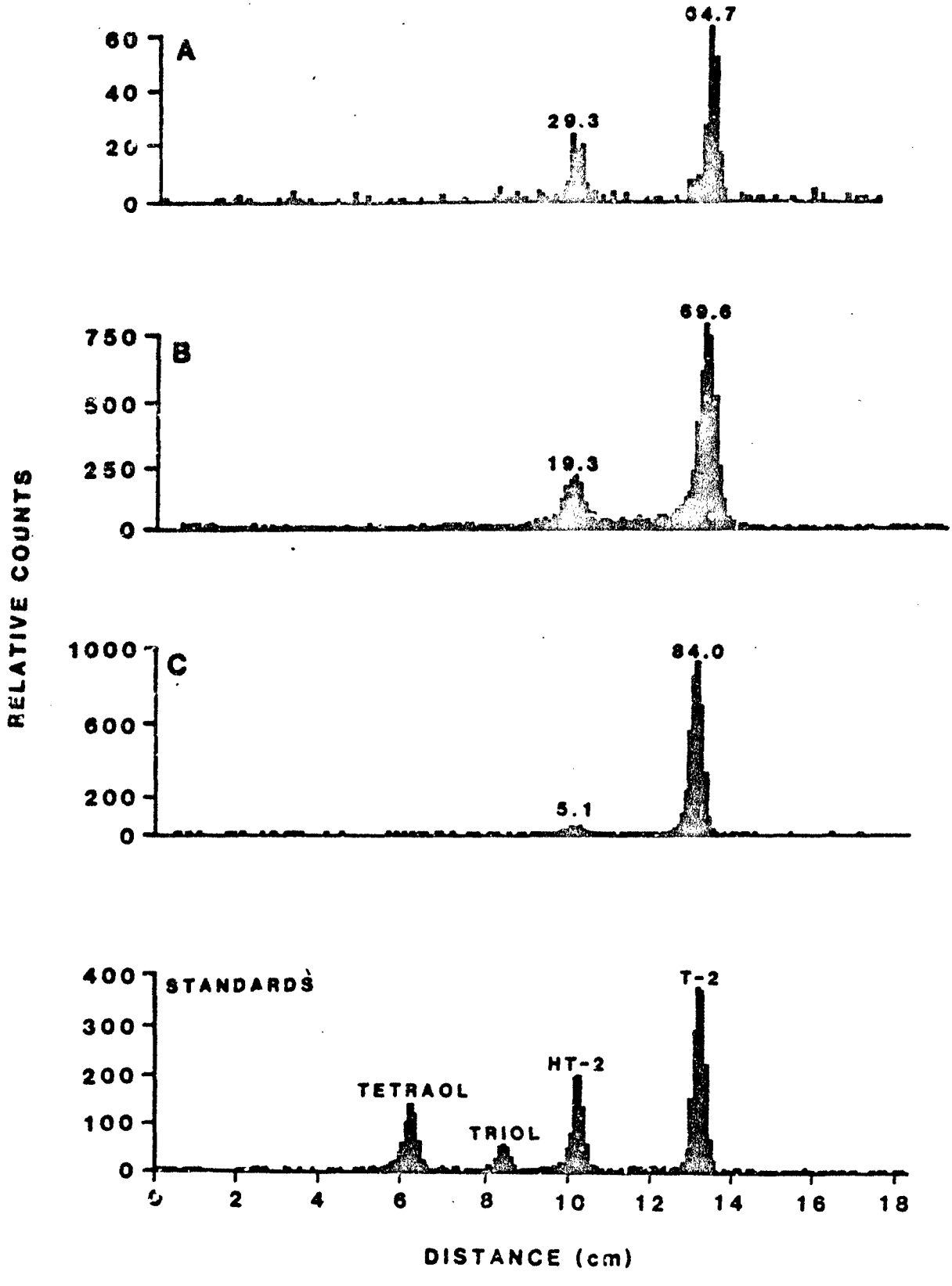
FIG. 7 Proposed pathway for metabolism of T-2 mycotoxin in rat hepatocytes. Solid arrows indicate conversions seen in hepatocytes, while dashed arrows indicate additional proposed pathways of T-2 metabolism in liver not seen in hepatocyte cell cultures. ME, microsomal enzyme systems; CE, cytosolic enzyme systems; 3'-OH-T-2, 3'-hydroxy T-2 toxin; 3'-OH-HT-2, 3'-hydroxy HT-2 toxin; DANS, deacetylneosolaniol.

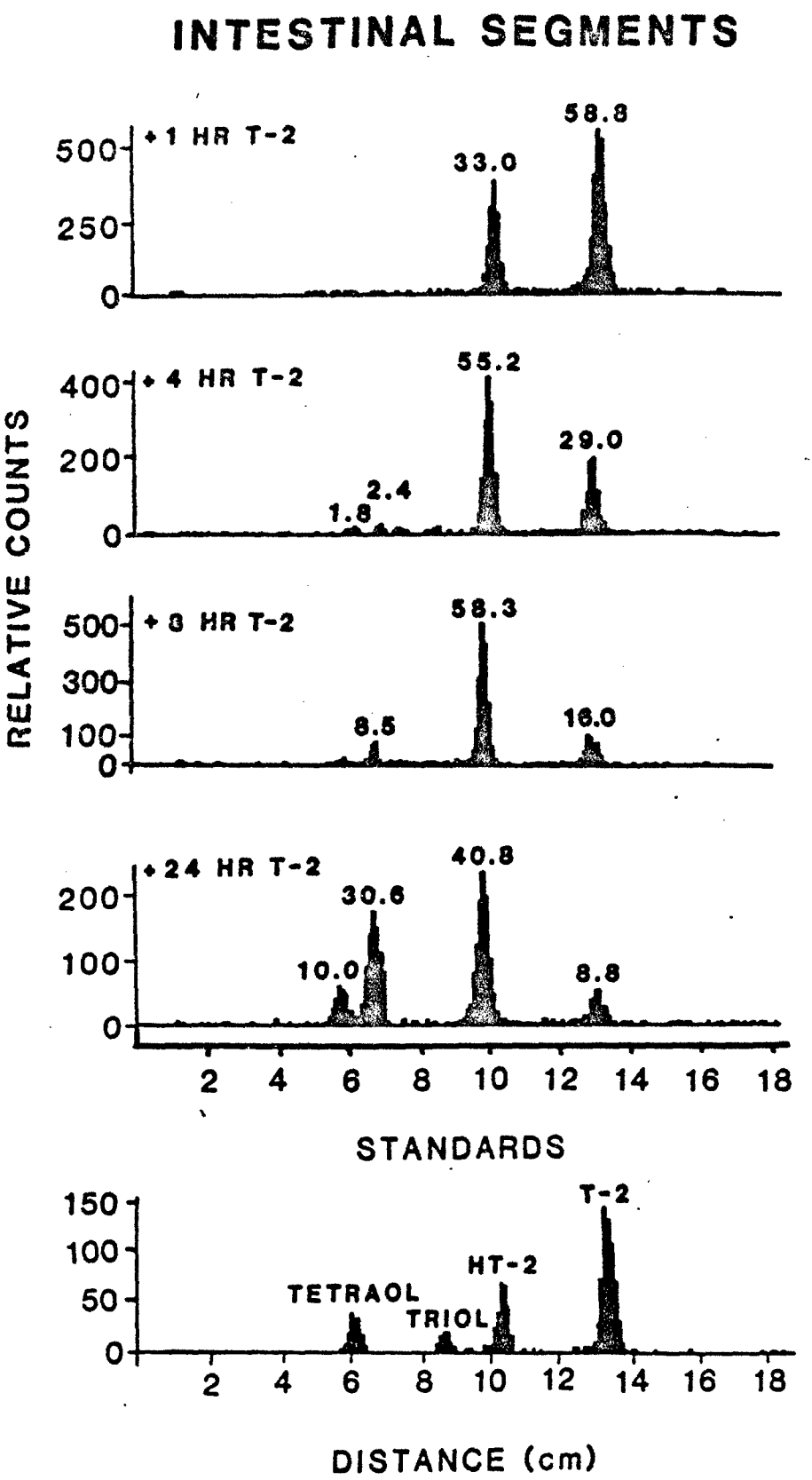
KEY WORDS

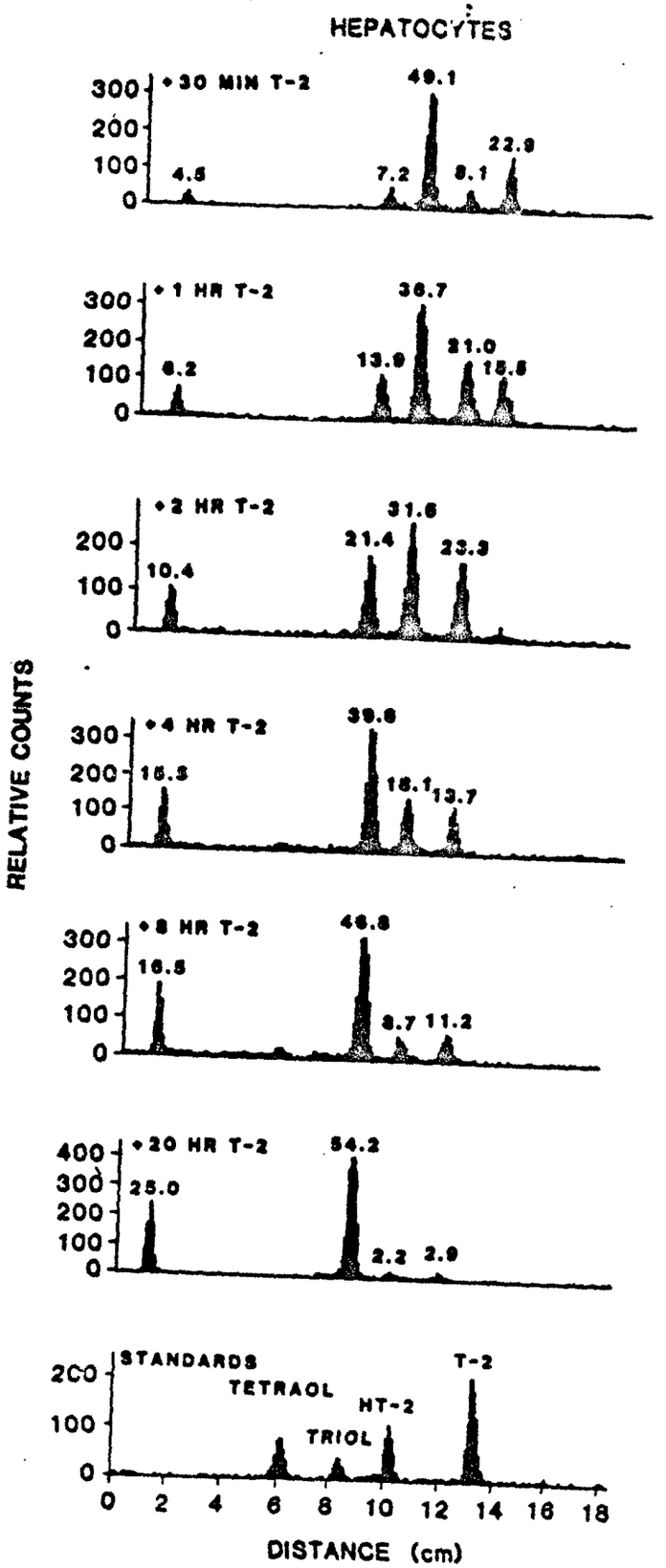
T-2 mycotoxin

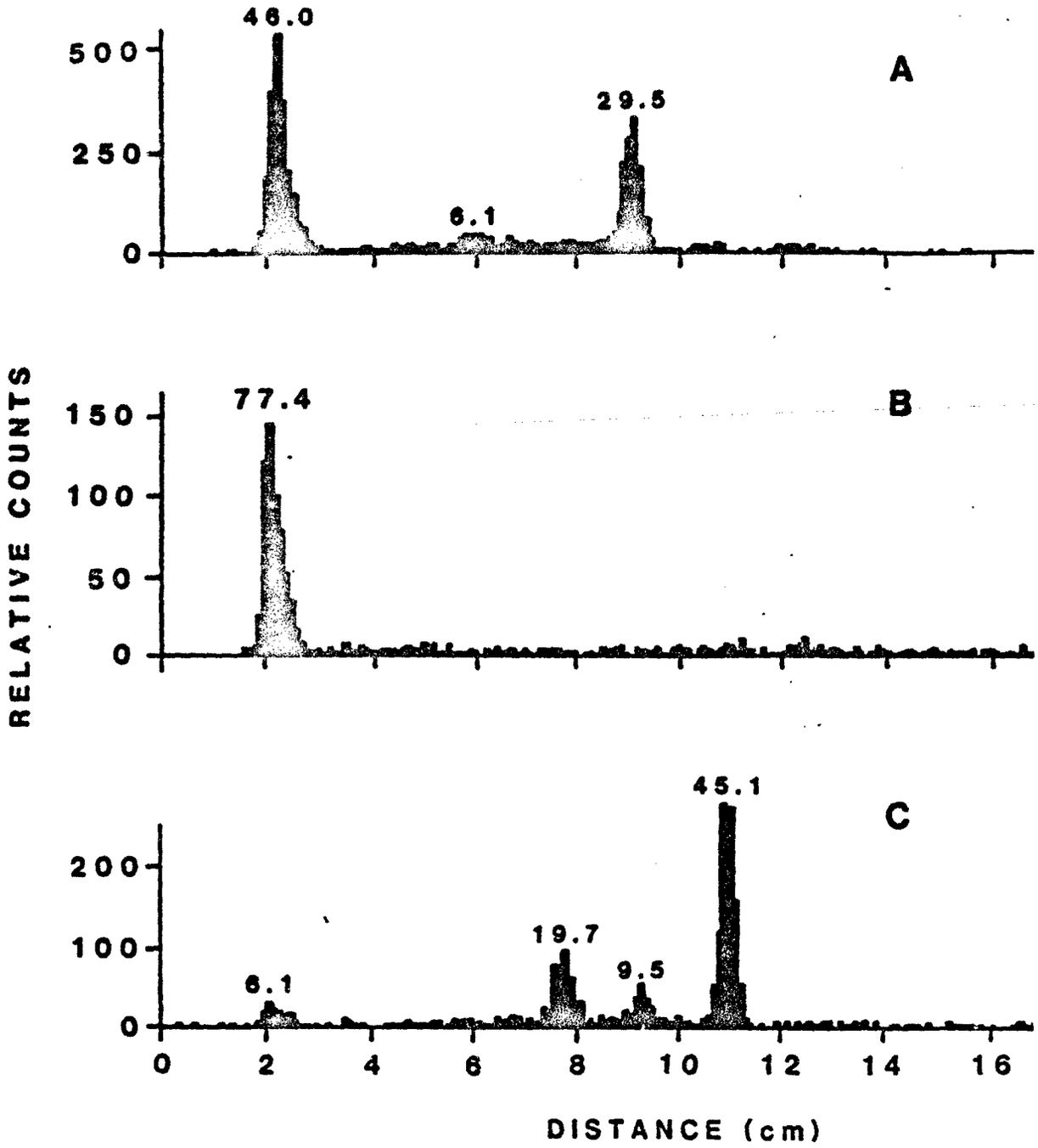
in vitro metabolism

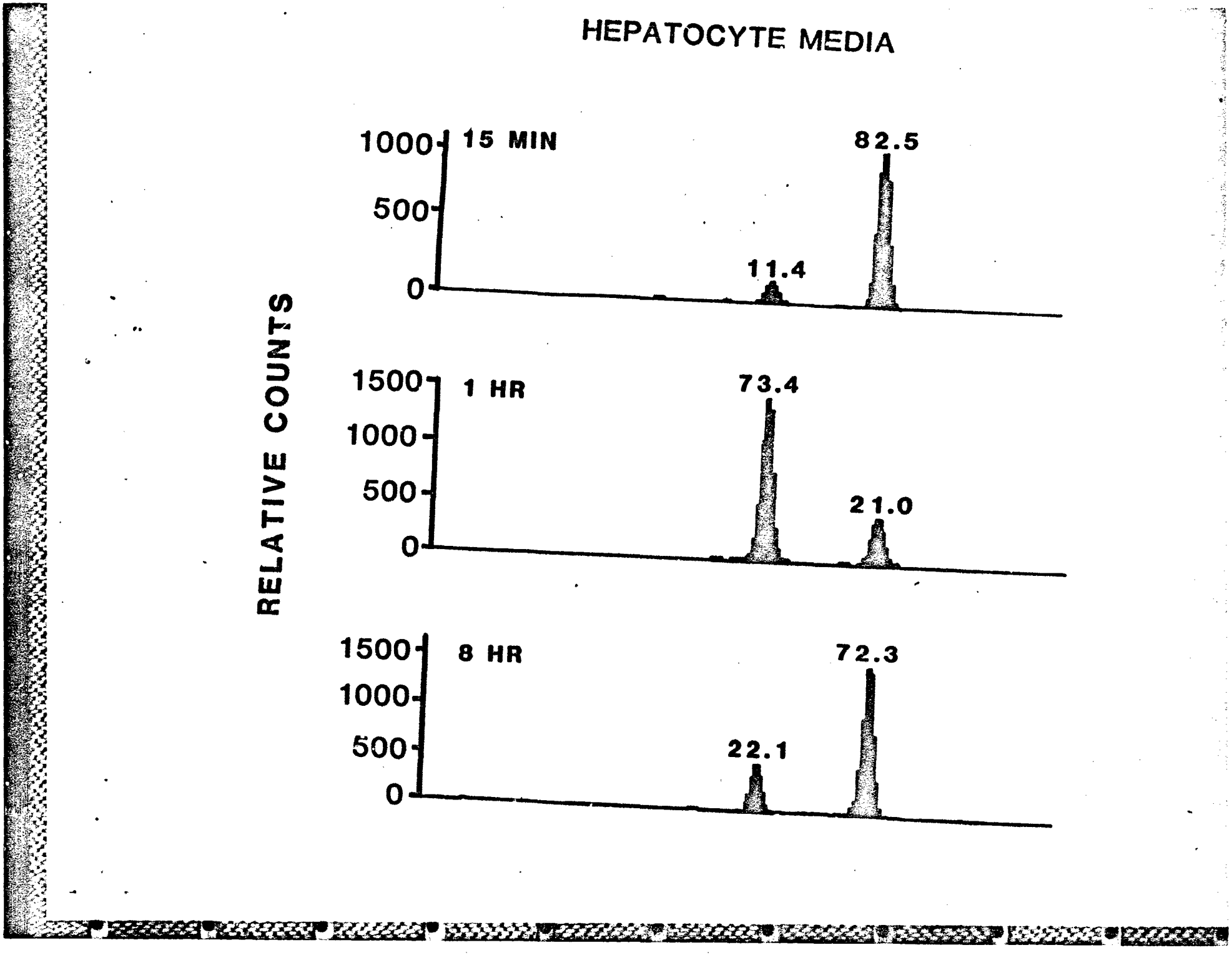
cultured cells

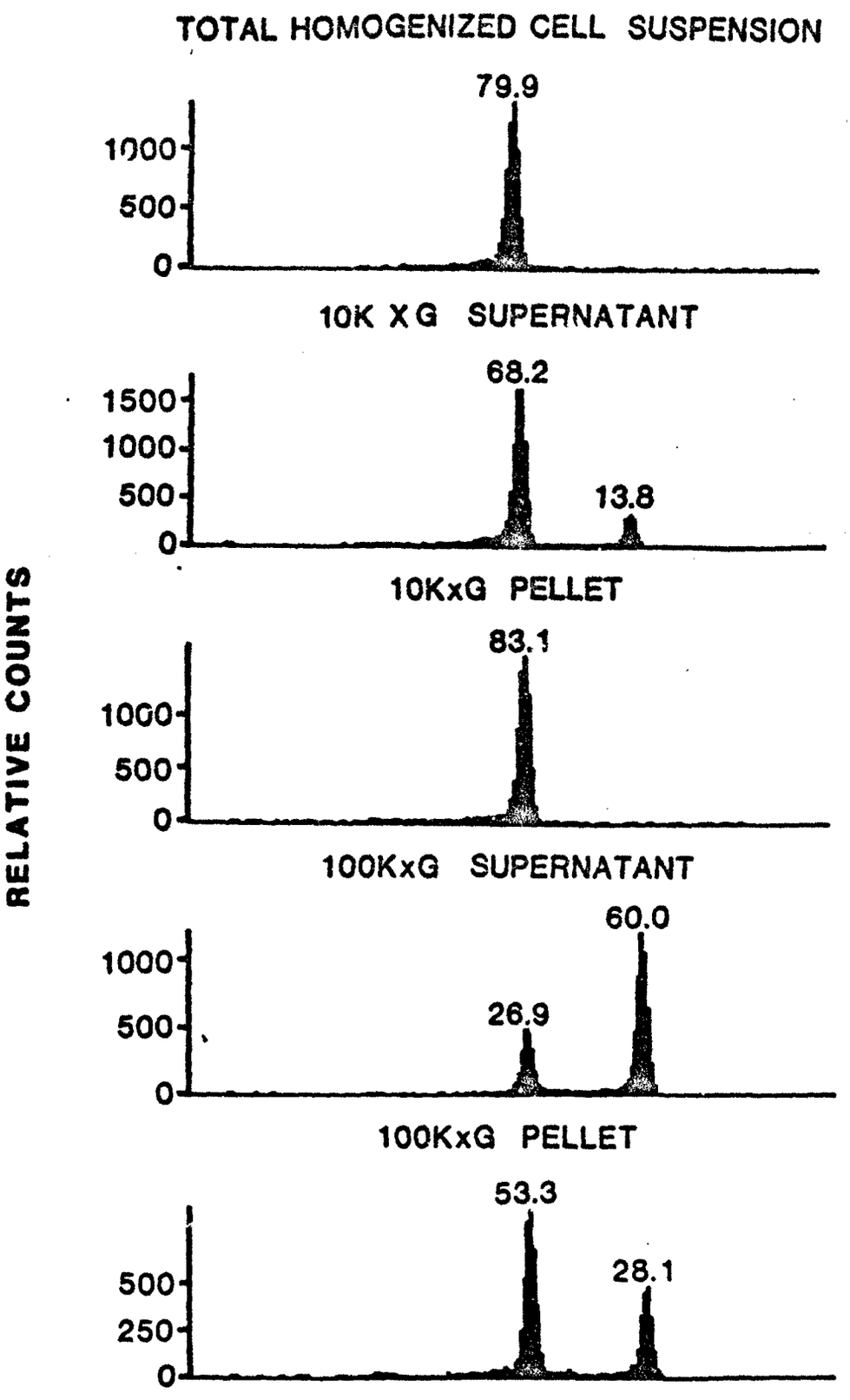

A
64.7
29.3
60
40
20
0
B
69.6
19.3
750
500
250
0
C
84.0
5.1
1000
600
200
0
STANDARDS
T-2
HT-2
TETRAOL
TRIOL
400
300
200
100
0
0
2
4
6
8
10
12
14
16
18
RELATIVE COUNTS
DISTANCE (cm)

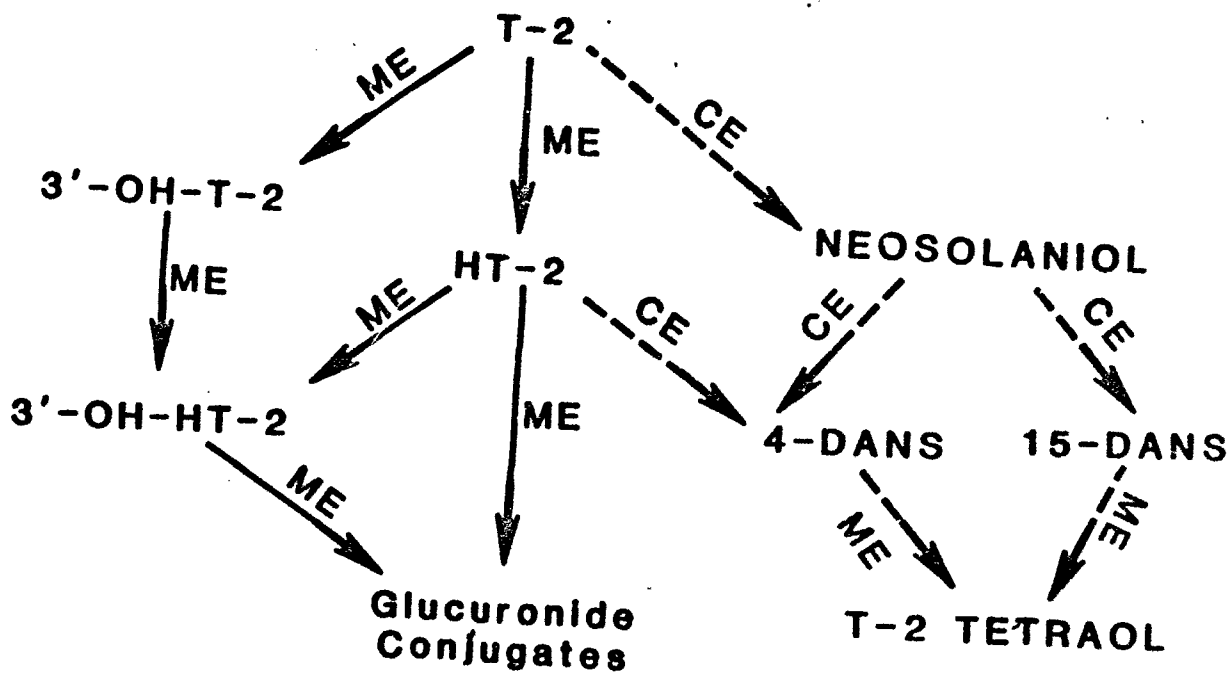

INTESTINAL SEGMENTS
+1 HR T-2
500
250
0
33.0
58.8
+4 HR T-2
400
200
0
1.8
2.4
55.2
29.0
+8 HR T-2
500
300
100
0
8.5
58.3
16.0
+24 HR T-2
200
100
0
10.0
30.6
40.8
8.8
2 4 6 8 10 12 14 16 18
STANDARDS
150
100
50
0
TETRAOL
TRIOL
HT-2
T-2
2 4 6 8 10 12 14 16 18
DISTANCE (cm)
RELATIVE COUNTS


HEPATOCYTES
• 30 MIN T-2
49.1
22.9
4.5
7.2
8.1
• 1 HR T-2
36.7
21.0
13.9
6.2
15.8
• 2 HR T-2
31.6
21.4
23.3
10.4
• 4 HR T-2
39.8
15.3
16.1
13.7
• 8 HR T-2
46.8
16.5
8.7
11.2
• 20 HR T-2
54.2
25.0
2.2
2.9
STANDARDS
TETRAOL
TRIOL
HT-2
T-2
RELATIVE COUNTS
DISTANCE (cm)


46.0
500
250
0
6.1
29.5
A
77.4
150
100
50
0
B
RELATIVE COUNTS
45.1
C
200
100
0
6.1
19.7
9.5
0
2
4
6
8
10
12
14
16
DISTANCE (cm)


HEPATOCYTE MEDIA
RELATIVE COUNTS
1000
500
0
15 MIN
82.5
11.4
1500
1000
500
0
1 HR
73.4
21.0
1500
1000
500
0
8 HR
72.3
22.1


TOTAL HOMOGENIZED CELL SUSPENSION
79.9
1000
500
0
10K XG SUPERNATANT
68.2
1500
1000
500
0
13.8
10KxG PELLET
83.1
1000
500
0
100KxG SUPERNATANT
60.0
1000
500
0
26.9
100KxG PELLET
53.3
500
250
0
28.1
RELATIVE COUNTS


T-2
ME
ME
CE
3'-OH-T-2
HT-2
NEOSOLANIOL
ME
ME
CE
CE
CE
3'-OH-HT-2
4-DANS
15-DANS
ME
ME
ME
ME
Glucuronide
Conjugates
T-2 TETRAOL